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Carbon-13 labelling strategy for studying the ATP metabolism in individual yeast cells by micro-arrays for mass spectrometry

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Experimental details

Materials

$^{13}C_2$-Ethanol, $^{13}C_6$-glucose, and LC-MS grade water were purchased from Sigma-Aldrich (St. Louis, MO, USA), while acetone and 9-aminoacridine were from Acros (Geel, Belgium). The high-density micro-arrays for mass spectrometry (MAMS) with 100-µm recipient sites were fabricated according to the method described previously (Urban *et al.*, *Lab Chip*, 2010, 10, 3206-3209). Briefly, indium tin oxide slides (Sigma-Aldrich) were first coated with a layer of polysilazane (EpoTec 611/2320; Eposint, Pfyn, Switzerland). Then, round-shaped recipient sites were created by localized ablation of the polysilazane layer using a picosecond laser (Nd:YAG-laser, SuperRapid; Lumera Laser, Kaiserslautern, Germany). The diameter of each recipient site was typically 100 µm. The recipient sites were arranged in a dense 'checkerboard'-like pattern.

Cell culture and sample preparation

For the experiment represented in **Figs. 1**-**3**, yeast (*Saccharomyces cerevisiae*, YSBN6 (mat a ho::HphMX4)) cells were grown in minimal defined medium with glucose as the sole carbon and energy source. The medium was prepared according to Verduyn *et al.* (*Yeast*, 1992, 8, 501-517) with 10-mM phthalate buffer to maintain pH at the value of 5. Liquid pre-cultures were inoculated with colonies from a YPD plate and grown until an OD_{600} of ~1.5. In the main culture, cells were grown in 50 mL of medium containing 1% glucose. Both cultures were incubated in a culture shaker at 30°C with 300 rpm. At an OD_{600} of ~1.5, cells were harvested and washed with minimal medium without a carbon source, and immediately re-suspended in 10 mL of minimal medium containing 0.5% $^{13}C_2$-ethanol with a start OD_{600} of 0.1. During the experiment with MAMS, the culture flask was placed in a thermostatted chamber (30°C), and the cell suspension was stirred with a magnetic stirrer. Samples were collected periodically with a pipette. A 1-mL aliquot of the suspension was normally mixed with 1 mL ice-cold water, and centrifuged at 3000 rcf for 5 min. The resulting pellet was resuspended in 2 mL of ice-cold water; following another centrifugation step, the pellet was finally resuspended in ~20 µL water, and the sample was stored on ice prior to the dispensing onto the MAMS target.

In some experiments, another *S. cerevisiae* strain (BY4741 (mat a his3Δ1/leu2Δ0/met15Δ0/ura3Δ0/rox3Δ::kanMX4)) was used (as indicated in the text and figure captions).

Except for using the isotope labels, the protocol for single-cell analysis by MAMS was as described in previous work (Urban *et al.*, *Lab Chip*, 2010, 10, 3206-3209). Briefly, the MAMS chip was placed on a stage of a modified inverted microscope. A transparent plastic chamber had previously been mounted on this stage to create an environment with controlled humidity. A stream of microdroplets, generated by an ultrasonic humidifier ("Repti Fogger"; Zoo Med Europa, Ekeren, Belgium), was directed into the chamber, and relayed onto the MAMS chip. A humidity sensor coupled with the humidity controller (Humidity Control II; Lucky Reptile, Waldkirch, Germany) was mounted inside the chamber to make sure that the humidity does not significantly drop down during the cell counting process. Cell suspension was applied to the MAMS surface with a micropipette. Excess of the suspension was removed, and the cells trapped in the individual recipient sites were counted using the microscope. The

MALDI matrix (9-aminoacridine) solution was applied to the dry surface of MAMS using a pulsed-action sprayer incorporating an ultrasonic scaler (UDS-J; Woodpecker Medical Instrument Company, Guilin, Guangxi, China).

A control experiment was carried out to verify low variation of the isotopic ratio (highly labelled (*m/z* 515) to poorly labelled (*m/z* 511) form of ATP) following resuspension of the cells in water; this experiment was done using samples composed of many yeast cells. During single-cell analysis by MAMS, numerous blank measurements were conducted by scanning empty recipient sites, to make sure the measurements are not biased by cross-contamination.

Mass spectrometry

We used the MALDI-TOF/TOF-MS 4800 *Plus* instrument (AB Sciex, Concord, ON, Canada) equipped with 355-nm solid-state laser. Measurements were conducted in the negative-ion reflectron mode. Single-cell measurements with MAMS were typically executed by firing 25-100 laser shots. MS/MS analysis was conducted using collision induced dissociation (CID) cell.

Data treatment

Spectral data from the experiments on single yeast cells on MAMS were initially exported to ASCII files, and further treated with custom scripts written in Free PASCAL (version 1.0.10 2009/04/10; B. Gábor, P. Muller, P. Vreman) and MATLAB (version 7.6.0.324 (R2008a), MathWorks, Natick, MA, USA). The PASCAL script read all the ASCII files containing mass spectra, and binned the ion intensities into 0.4-mass unit bins. Due to unequal numbers of data points per bin, the sums were divided by numbers of data points in each bin to obtain average signal strength. Following normalization (dividing signal intensities by the sum of signal intensities in the 11 bins, from 506 to 516), the bin values corresponding to the 11 isotopic forms of ATP (*m/z* 506 – non-labelled, till *m/z* 516 – fully labelled) – recorded for each single-cell spectrum – were input to a data matrix and exported to a new ASCII file. This file was then loaded to MATLAB. Principal component analysis was conducted using the MATLAB command *princomp*, and the output was subsequently plotted using the command *biplot*. Other data were treated and displayed with the Origin Pro software (version 8; Origin Lab Corporation, Northampton, MA, USA), or Excel (2007; Microsoft, Redmond, WA, USA).

In **Fig. 3**, 253 data points, corresponding to single-cell mass spectra obtained for various incubation times (1 h – 61, 10 h – 19, 24 h – 100, 48 h – 73), are included. The experiment was carried out three times with minor modifications. It should be pointed out that, due to the limited mass resolution and the binning procedure, some low background peaks – which are barely resolved from the metabolite peaks (*cf.* **Figs. 2** and **S1**) – might have been counted as metabolite signals. However, due to the character of these peaks (random or constant noise), this should not bias the clustering of data points in **Fig. 3**; in fact, only spectra with ATP peaks, which have sufficiently high signal-to-noise ratio were included in the analysis.

Additional figures

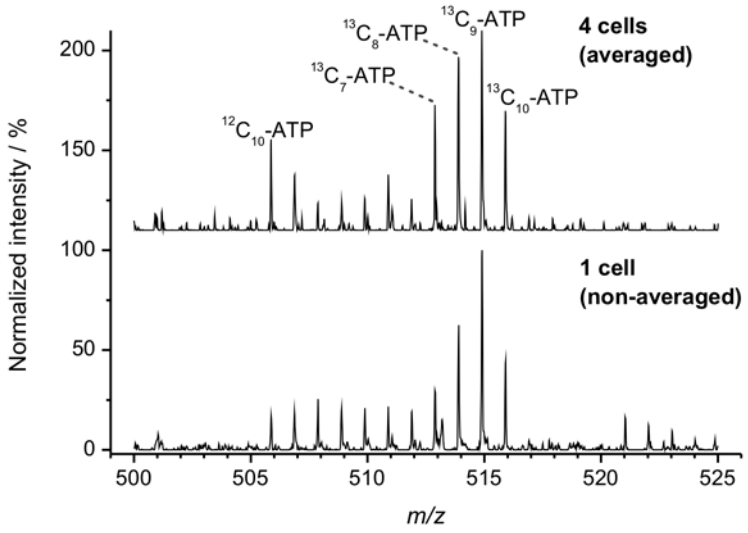

Fig. S1 Negative-ion MALDI mass spectra of 4 cells (averaged) and 1 cell, after 24-h yeast (strain YSBN6 (mat a ho::HphMX4)) culture in 0.5% $^{13}C_2$-ethanol medium. MAMS used with 9-aminoacridine as MALDI matrix.

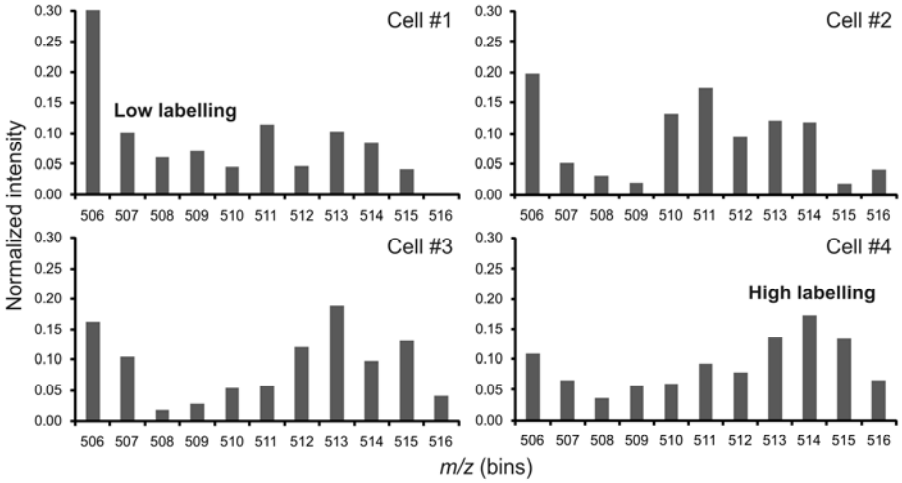

Fig. S2 Bar graphs corresponding to the raw mass spectra presented in **Fig. 2**. Spectral data have been binned and normalized as described above.

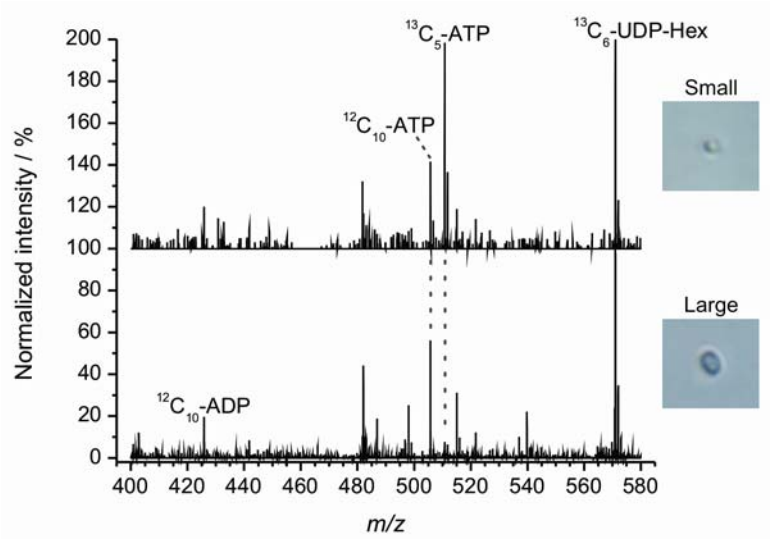

Fig. S3 Differences in the ratio of partly labelled and non-labelled ATP in the yeast cells of different size. Yeast strain: BY4741 (mat a his3Δ1/leu2Δ0/met15Δ0/ura3Δ0/rox3Δ::kanMX4); cells of this strain are (on average) larger than the cells of wild-type strain. Cells were incubated with 2% $^{13}C_6$-glucose at 30°C for 30 min. prior to the analysis by negative-ion MALDI-MS using MAMS and 9-aminoacridine as MALDI matrix. UDP-Hexose is fully labelled in both cells, while the labelled/non-labelled ATP ratio is higher in the small cell relative to the large cell. The rationale for this experiment was the following: In order to verify which is the reason for a low or high level of ATP labelling in a particular cell (*cf.* **Fig. 2**), one may correlate the spectral information with sizes of the analyzed cells (estimated from the optical microscope images). However, systematic correlation of image and spectral data will require further upgrade of the auxiliary instrumentation (automated microscopy, and high-throughput image analysis), and is beyond the scope of the present study.

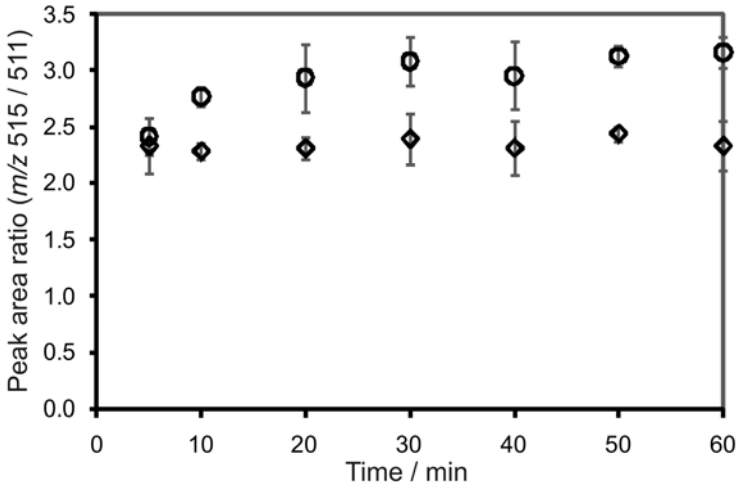

Fig. S4 Changes in isotopic ratio of ATP (peak area ratio for *m/z* 515/511: two relatively abundant isotopic forms of ATP present in the sample) after resuspending the isotopically labelled yeast cells in water. Yeast grown on $^{13}C_2$-EtOH (~ 24 h) were resuspended in cold water (after centrifugation) and stored on ice (◇) or at room temperature (○). Samples were taken for analysis by negative-ion mode MALDI-MS (using 9-aminoacridine as matrix), and ratios of the two selected isotopic forms were determined. Error bars refer to standard deviation (n = 4). Clearly, when the cells are stored on ice, the peak ratio (*m/z* 515/511) remains constant during 1 h. When the cells are stored at room temperature, the peak ratio slightly increases during the first 20 min. of incubation (probably due to the ongoing synthesis of ATP using residue $^{13}C_2$-EtOH or labelled intermediates), but later on, a plateau is reached. This result is explained in the following way: Any alteration of the $^{13}C/^{12}C$ composition of ATP is caused by the incorporation of carbon-13 to ribose and adenine moieties. This process involves numerous enzymes and intermediate metabolites. Therefore, it is a relatively slow process when compared to the hydrolysis of ATP to ADP, or phosphorylation of ADP (transformations catalyzed by single enzymes).

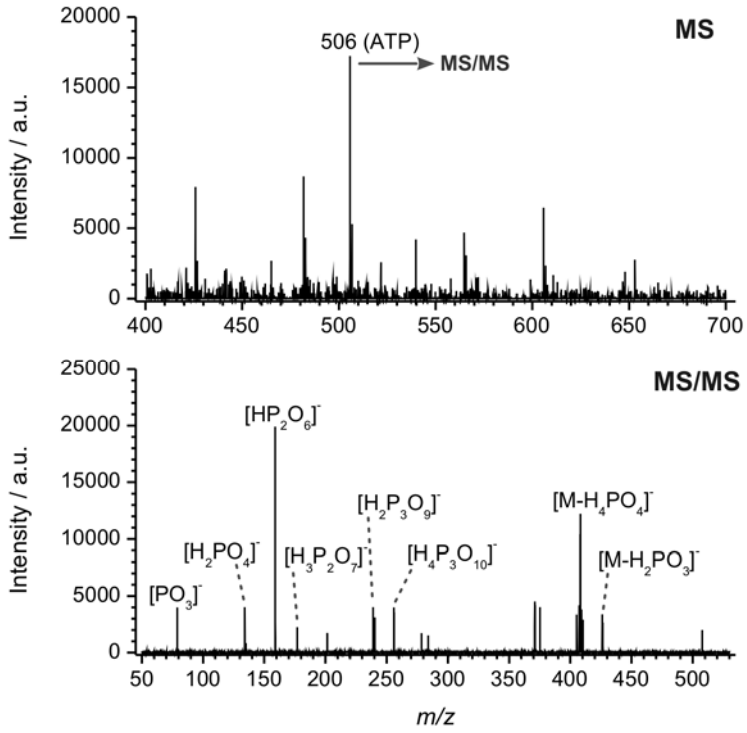

Fig. S5 Mass spectrum of single yeast cell (non-labelled), and a corresponding single-cell tandem mass spectrum for the *m/z* 506 as parent ion. Yeast strain: BY4741 (mat a his3Δ1/leu2Δ0/met15Δ0/ura3Δ0/rox3Δ::kanMX4); cells of this strain are (on average) larger than the cells of wild-type strain. Fragment assignment is based on the article by Sun *et al.* (*Anal. Chem.*, 2007, 79, 6629-6640).

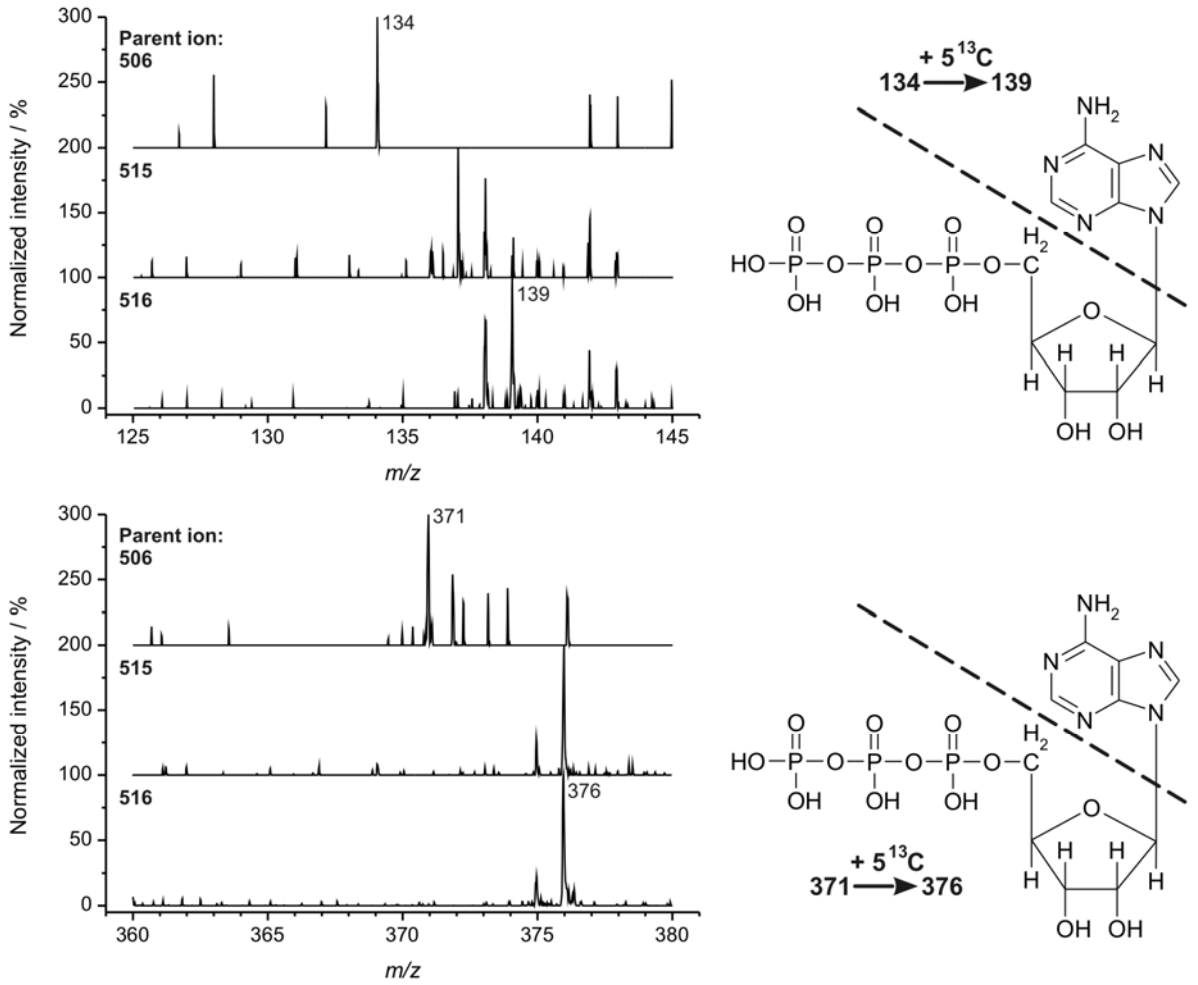

Fig. S6 Sections of negative-ion mode MALDI tandem mass spectra revealing incorporation of carbon-13 into the adenosine and ribose groups of ATP. The experiment was carried out on samples composed of multiple yeast cells cultured on $^{13}C_2$-ethanol medium. Note that the appearance of two isotopic forms of fragment ions may be due to distribution of carbon-13 label between adenine and ribose group within the population of parent ion, as well as inability to precisely select single parent ions prior to CID fragmentation. Fragment assignment is based on the article by Sun *et al.* (*Anal. Chem.*, 2007, 79, 6629-6640).